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Site-directed Mutagenesis of Cysteine Residues in the Pro Region of the Transforming Growth Factor β1 Precursor

EXPRESSION AND CHARACTERIZATION OF MUTANT PROTEINS*

(Received for publication, March 27, 1989)

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Three cysteine residues are located in the pro region of th transforming growth factor β1 (TGF-β1) precursor at amino acid positions 33, 223, and 225. Previous studies (Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) *Mol. Cell. Biol.* 8, 4162–4168) with purified recombinant TGF-β1 (rTGF-β1) precursor produced by Chinese hamster ovary (CHO) cells revealed that Cys-33 can form a disulfide bond with at least 1 cysteine residue in mature TGF-β1, contributing to the formation of a 90–110-kDa protein. W now show that Cys-223 and Cys-225 form interchain disulfide bonds. Site-directed mutagenesis was us d to change these Cys codons to Ser codons, and mutant constructs were transfected into COS cells. Analysis of recombinant proteins by immunoblotting showed that by substituting Cys-33 the 90–110-kDa protein is not formed, and thus, more mature dimer (24 kDa) is obtained, corresponding to a 3- to 5-fold increase in biological activity. Substitution of Cys-223 and/ r Cys-225 resulted in near wild-type levels of mature TGF-β1. Furthermore, cells transfected with plasmid coding for Ser at positions 223 and 225 expressed only monomeric precursor proteins and re l ased bioactive TGF-β1 that did not require acid activati n, suggesting that dimerization of the precursor pro r gion may be necessary for latency.

Transforming growth factor β1 (TGF-β1)[1] belongs to a family of polypeptides that regulate cell growth and differentiation (for reviews see Refs. 1–3). Although originally defined by its ability to stimulate the anchorage-independent growth of nontumorigenic fibroblasts (4), TGF-β1 has been shown to inhibit proliferation of a variety of cells including endothelial cells (5, 6), T and B lymphocytes (7–9), and keratinocytes (10). This polypeptide's diverse effects also include stimulation of fibronectin and collagen synthesis and secretion (11, 12), induction of squamous cell differentiation (13), and inhibition of myogenic differentiation (14).

Biologically active TGF-β1 is a 24-kDa homodimer interlinked by at least one disulfide bond (15, 16). Analyses of cDNA clones coding for human (17), simian (18), and murine (19) TGF-β1 have predicted that the 112-residue chain of TGF-β1 is proteolytically cleaved from the COOH terminus of a 390-amino acid prepro-TGF-β1. The precursor contains a typical hydrophobic signal peptide sequence and three potential *N*-linked glycosylation sites.

Simian TGF-β1 cDNA has been expressed to high levels in CHO cells, resulting in the secretion of both precursor and mature forms of TGF-β1 (20). Subsequent studies have revealed that the rTGF-β1 precursor is glycosylated at all three sites and phosphorylated at mannose residues (21, 22). Recent experiments suggest that carbohydrate addition to the pro region of the TGF-β1 precursor is necessary for transport and secretion of mature polypeptide.[2] The pro region of the precursor may also be important in the regulation of TGF-β1. Several studies (23–25) have shown that most cell types secrete TGF-β1 in a biologically inactive form. rTGF-β1 is also secreted in a latent form which can be activated by acidification (20). In addition, a latent complex has been isolated from platelets (26, 27) in which mature TGF-β1 is noncovalently associated with the pro region and an unidentified protein, and further studies (28) have indicated that carbohydrate structures in the pro region are necessary for latency.

Interestingly, purified recombinant precursor was shown to consist of pro-TGF-β1 (residues 30–390; *a* in Fig. 2*A*), the pro region of the precursor (residues 30–278; *b* in Fig. 2*A*), and mature TGF-β1 (residues 279–390; *c* in Fig. 2*A*) interlinked by disulfide bonds (29). The most abundant form of the rTGF-β1 precursor was found to contain two homodimeric subunits bridged by intersubunit disulfide bonds between Cys-33 in the TGF-β1 pro region subunit and cysteine residues in the mature TGF-β1 subunit to form a 90–110-kDa protein. This suggests that more biologically active TGF-β1 dimer might be generated if Cys-33 was substituted, allowing more of the processed mature TGF-β1 to be released from the precursor complex.

In addition to Cys-33, 2 other cysteine residues are located in the pro region at positions 223 and 225. In this report, we present assignment of two intermolecular disulfide bonds between residues 223 and 225, respectively. We also examine the significance of these 3 cysteines in dimerization, processing, and latency of TGF-β1 through the use of site-directed mutagenesis followed by expression of mutant constructs in COS cells.

EXPERIMENTAL PROCEDURES

Characterization of CNBr Peptide M(134–253)$_2$—rTGF-β1 precursor synthesized by CHO cells (20) was purified by gel permeation chromatography, cleaved with CNBr, and the peptides purified fol-

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[1] The abbreviations used are: TGF-β, transforming growth factor β; rTGF-β1, recombinant TGF-β1; SDS, sodium dodecyl sulfate; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography.

[2] X. Sha, A. M. Brunner, A. F. Purchio, and L. E. Gentry, manuscript in preparation.

lowing previously described methods (29). Cys-containing peptides were detected after reduction with tributylphosphine and coupling with ammonium 7-fluorobenzo-2-oxo-1,3-diazole-4-sulfonate (30) and purified to homogeneity by reversed-phase HPLC on a μBondapak C_{18} column (Waters Associates Inc., Milford, MA) (29).

Peptides were separated on 15% SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue R-250 as described (31). Disulfide-linked peptides were analyzed with an Applied Biosystems 475A Sequencer. Phenylthiohydantoins were separated on an Applied Biosystems 120A PTH-analyzer as described (29).

Site-directed Mutagenesis—Mutagenesis was performed using the Amersham *in vitro* mutagenesis system, according to the manufacturer's protocol. The template for mutagenesis consisted of the *Pst*I-*Eco*RI fragment from pTGF-β-2 (18), coding for simian TGF-β1 precursor, inserted into the *Pst*I and *Eco*RI sites of M13mp18. The oligonucleotides used to construct the mutants were synthesized on an Applied Biosystems DNA synthesizer and are shown in Fig. 2*B*. Each encodes a Ser residue in place of the indicated Cys residue. In each case, 5–10 plaques were screened for the desired mutation by single track sequencing and the final mutant characterized by further sequencing using the dideoxy chain termination method (32).

Construction of Expression Vectors and Transient Expression—The mutant genes were subcloned into the expression vector πH3M (33), lacking the polylinker between XhoI sites. πH3M was cleaved with *Xho*I, filled in with Klenow fragment, and digested with *Hin*dIII. Each mutant cDNA was excised from the M13-derived replicative form by digestion with *Eco*RI, followed by repair to blunt ends with Klenow fragment and digestion with *Hin*dIII, and then ligated into the *Hin*dIII-*Xho*I (blunt) vector. Expression plasmid pTGF-$\beta1^{S33}$ codes for the protein prepro-TGF-$\beta1^{S33}$, containing a serine at position 33 of the TGF-β1 precursor in place of the wild-type cysteine residue. Similarly, pTGF-$\beta1^{S223}$, pTGF-$\beta1^{S225}$, and pTGF-$\beta1^{S223/225}$ code for prepro-TGF-$\beta1^{S223}$, prepro-TGF-$\beta1^{S225}$, and prepro-TGF-$\beta1^{S223/225}$, respectively, with superscripts indicating the positions where cysteine residues have been replaced with serines.

For transient expression of the mutant gene products, the plasmids were transfected into COS cells by the DEAE-dextran/chloroquine method as described (34). Each transfection was performed at least three times. COS cells were transfected at 30–60% confluency with 25 μg of DNA/100-mm plate in a total volume of 5 ml for 2.5 h at 37 °C. After a 48-h incubation in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, the medium was replaced with serum-free Dulbecco's modified Eagle's medium. Transfected cells were incubated an additional 72 h prior to supernatant collection.

Analysis of Mutant Proteins—Serum-free supernatants from transfected COS cells were dialyzed against 0.2 M acetic acid. TGF-β1 activity was determined by growth inhibition of mink lung epithelial cells (MvLu; CCL-64: American Type Culture Collection) as described previously (35). Highly purified recombinant TGF-β1 (29) synthesized by CHO cells and possessing the same specific activity as natural TGF-β1 was used as a standard. Typically, 80–120 pg of TGF-β1/ml resulted in 50% inhibition.

Secreted TGF-β1 proteins were analyzed by immunoblotting (20, 36). Briefly, 0.8 ml of acid-dialyzed supernatant was lyophilized, resuspended in 50 μl of sample buffer, fractionated on 7.5–17.5% gradient SDS-polyacrylamide gels, and transferred to nitrocellulose. TGF-β1 proteins were detected with anti-peptide antibodies specific for mature TGF-β1 (anti-TGF-$\beta1_{369-381}$) or the pro region of the TGF-β1 precursor (anti-TGF-$\beta1_{81-94}$) (20).

RESULTS

TGF-β1 Precursor Cysteine Residues 223 and 225 Form Interchain Disulfide Bonds—Purified rTGF-β1 precursor was cleaved at methionine residues with CNBr, and the resulting peptides were purified by gel permeation chromatography. Cys-containing peptides were detected according to the procedure of Sueyoshi *et al.* (30) and purified to homogen ity by reversed-phase HPLC. Edman degradation data located the positions of the CNBr peptides in the sequence of the TGF-β1 precursor. Peptide $M(134–253)_2$ contained a 2 half-cystine residues, as determined by amino acid analysis, but lacked free sulfhydryl groups, as determined with a fluorescent thiol-specific reagent under nonreducing conditions. Disulfide bridges in $M(134–253)_2$ were assigned by demonstrating the dimeric character of this peptide. As shown in Fig. 1, the size

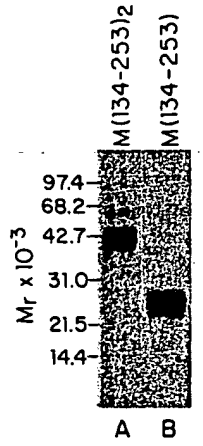
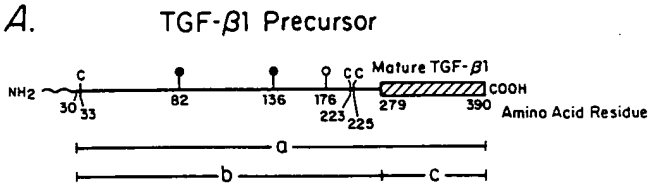


FIG. 1. **SDS-polyacrylamide gel analysis of purified cyanogen bromide peptide M(134–253).** M(134–253) was fractionated on a 15% SDS-polyacrylamide gel under nonreducing (A) or reducing (B) conditions and stained with Coomassie Brilliant Blue R-250. Size markers are indicated in kilodaltons.



B.

Mutant	Oligonucleotide
TGF-$\beta1^{S33}$	ACTATCCACC**AGC**AAGACTAT
TGF-$\beta1^{S223}$	TAGCGCCCAC**AGC**TCCTGTGA
TGF-$\beta1^{S225}$	CACTGCTCC**TCT**GACAGCAAA
TGF-$\beta1^{S223/225}$	TAGCGCCCAC**AGC**TCC**TCT**GACAGCAAA

FIG. 2. *A*, line diagram of prepro-TGF-β1. Pro-TGF-β1, the pro region of the precursor, and mature TGF-β1 are indicated by *lines a*, *b*, and *c*, respectively. Cys (*C*) residues in the pro region are indicated. Mannose 6-phosphate-containing sites (♀) and nonphosphorylated sites (♀) of *N*-linked glycosylation are shown. *B*, TGF-β1 precursor mutants. Superscripts indicate the amino acid position of the Ser substitutions. Unmatched nucleotides are in ***bold print*** and new Ser codons are *underlined*.

of $M(134–253)_2$ was estimated at 41 kDa under nonreducing conditions, whereas under reducing conditions M(134–253) had a mass of 24 kDA. The change in size of $M(134–253)_2$ following reduction identifies two intermolecular disulfide bonds linking identical chains, consistent with a single amino acid sequence for peptide $M(134–253)_2$ and the determination of 2 half-cystine residues by amino acid analysis. The pro region of rTGF-β1 precursor secreted by CHO cells has been shown to be glycosylated at Asn-82, −136, and −176 (21, 22). Therefore, the observed heterogeneity of $M(134–253)_2$ on SDS-polyacrylamide gels (Fig. 1) most likely reflects glycosylation at Asn-136 and Asn-176.

Analysis of Proteins Encoded by Mutant TGF-β1 Genes—Three cysteine residues are located in the pro region of the TGF-β1 precursor at amino acid positions 33, 223, and 225. A line diagram of the TGF-β1 precursor is displayed in Fig. 2*A*. Using simian TGF-β1 cDNA as a template, each Cys codon was separately changed to a Ser codon by site-directed mutagenesis. Oligonucleotides used to direct the mutagenesis are shown in Fig. 2*B*. In each case, a single base pair change

accomplished the Cys to Ser codon switch. A double mutant in which both Cys-223 and Cys-225 were changed to Ser codons was also constructed.

The mutant TGF-β1 cDNAs were inserted into the expression vector πH3M (33) and transiently expressed in COS cells. Serum-free supernatants from the transfected cells were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with a mixture of antibodies specific for the pro and mature regions of the TGF-β1 precursor (Fig. 3). Under reducing conditions (Fig. 3*A*), the mutant proteins appeared identical to the wild type. Antibodies detected the 12-kDa monomer (*c* in Fig. 2*A*) as well as 44–56-kDa and 30–42-kDa precursors forms (*a* and *b* in Fig. 2*A*). In addition, the immunoblot shown in Fig. 3*A* indicates that the absolute amount of secreted TGF-β1 proteins was not significantly affected by the mutations.

Differences between mutant and wild-type TGF-β1 proteins were readily apparent when examined under nonreducing conditions (Fig. 3*B*). Mature TGF-β1 dimer (24 kDa) was present in all transfectant supernatants. However, cells transfected with pTGF-$\beta1^{S33}$ yielded increased levels of the 24-kDa dimer compared to wild-type transfectants (Fig. 3*B*, *lanes 3* and *4*), whereas pTGF-$\beta1^{S223}$, pTGF-$\beta1^{S225}$, and pTGF-$\beta1^{S223/225}$ transfectants produced near wild-type levels. This increase in 24-kDa dimer did not appear to be a result of increased protein synthesis/secretion or increased cleavage of mature TGF-β1 from pro-TGF-β1, since immunoblotting under reducing conditions indicated that pTGF-β1 and pTGF-$\beta1^{S33}$ transfectants secreted approximately equal amounts of both mature TGF-β1 and precursor forms (Fig. 3*A*).

In addition, the TGF-$\beta1^{S33}$ proteins did not form the 90–110-kDa precursor complex as did wild-type proteins (Fig. 3*B*). Instead, a 130 to 150-kDa and 75 to 85-kDa species were seen (Fig. 3*B*, *lane 4*). As shown in Fig. 4, the TGF-$\beta1^{S33}$ 130–150-kDa proteins were recognized by both pro region- and mature-specific antibodies and most likely represent a dimeric complex of pro-TGF-$\beta1^{S33}$ linked by disulfide bonds. The

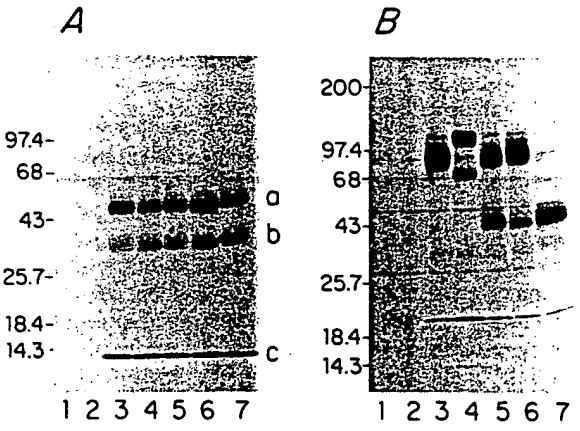


FIG. 3. **Immunoblot of TGF-β1 mutant proteins secreted by transfected COS cells.** Transfected cells were incubated in serum-free media (5 ml/100-mm dish) 48 h post-transfection for 72 h. Serum-free supernatants were collected, dialyzed *versus* 0.2 M acetic acid, and 0.8 ml of each supernatant was fractionated on 7.5–17.5% SDS-polyacrylamide gels under reducing (*A*) or nonreducing (*B*) conditions. Immunoblots were probed with a mixture of pro region (anti-TGF-$\beta1_{81-94}$) and mature (anti-TGF-$\beta1_{369-381}$) specific anti-peptide antibodies. COS cells were transfected with vector (πH3M) only (*lane 2*, *A* and *B*) or with vector encoding TGF-β1 (*lane 3*, *A* and *B*), TGF-$\beta1^{S33}$ (*lane 4*, *A* and *B*), TGF-$\beta1^{S223}$ (*lane 5*, *A* and *B*), TGF-$\beta1^{S225}$ (*lane 6*, *A* and *B*) and TGF-$\beta1^{S223/225}$ (*lane 7*, *A* and *B*). *Lane 1* contains supernatant from nontransfected COS cells. Size standards are indicated in kilodaltons. Results are representative of three separate experiments.

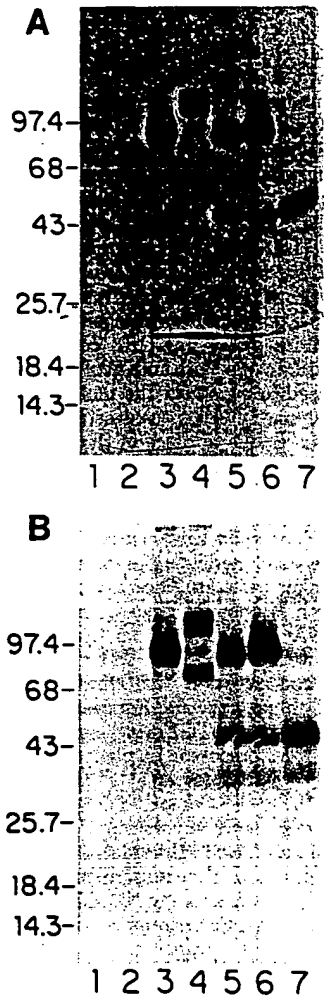


FIG. 4. **Identification of mutant TGF-β1 precursor and mature proteins.** Supernatants from COS cells transfected with πH3M (*lane 2*, *A* and *B*), pTGF-β1 (*lane 3*, *A* and *B*), pTGF-$\beta1^{S33}$ (*lane 4*, *A* and *B*), pTGF-$\beta1^{S223}$ (*lane 5*, *A* and *B*), pTGF-$\beta1^{S225}$ (*lane 6*, *A* and *B*), or pTGF-$\beta1^{S223/225}$ (*lane 7*, *A* and *B*) were collected and processed as described in the legend to Fig. 3. Samples were fractionated on nonreducing 7.5–17.5% SDS-polyacrylamide gels and immunoblotted with antibodies specific for mature (*A*) sequences (anti-TGF-$\beta1_{369-381}$) or the pro region (*B*) (anti-TGF-$\beta1_{81-94}$). *Lane 1* contains nontransfected COS cell supernatant. Size standards are indicated in kilodaltons.

rTGF-β1 proteins secreted by COS cells transfected with plasmids encoding wild-type or mutant TGF-β1 precursor appear identical when analyzed under reducing conditions (Fig. 3*A*); therefore, the 130–150-kDa complex observed under nonreducing conditions (Fig. 3*B*, *lanes 3* and *4*) most likely results from structural changes in the molecules due to disulfide bonding. The 75–85-kDa polypeptide was only detected by pro region-specific antibody (Fig. 4*B*, *lane 4*) and probably represents a dimer of the pro region of the TGF-$\beta1^{S33}$ precursor.

In contrast, mutant proteins encoded by pTGF-$\beta1^{S223}$ and pTGF-$\beta1^{S225}$ formed the 90–110-kDa precursor complex (*lanes 5* and *6* in Figs. 3B, 4*A*, and 4*B*), although some monomeric precursor forms were also present. The slight difference in molecular weight between the pro-TGF-$\beta1^{S223}$ and pro-TGF-$\beta1^{S225}$ 90–110-kDa precursor complexes (Fig. 4*B*, *lanes 5* and *6*) may be due to variations in glycosylation or differences in folding due to altered disulfide bonding. Antibodies specific for the pro region of the TGF-β1 precursor detected 44–56-kDa and 30–42-kDa proteins (Fig. 4*B*, *lanes 5* and *6*), whereas

TABLE I

Amount of bioactive TGF-β1 secreted by COS cell transfectants

COS cells were transfected with plasmids encoding TGF-β1 and variants; 48 h post-transfection, supernatants were replaced with serum-free medium; 72 h later, conditioned media were collected and assayed directly (−acid) or following dialysis *versus* 0.2 M acetic acid (+acid) for growth inhibition of CCL64 cells as described under "Experimental Procedures."

	+acid	−acid
	ng/ml	
TGF-β1	90.3	4.7
TGF-β1^{S33}	304.8	12.5
TGF-β1^{S223}	98.4	20.3
TGF-β1^{S225}	77.9	11.2
TGF-β1$^{S223/225}$	87.9	65.3

antibody specific for mature sequences detected only the 44–56-kDa species (Fig. 4*A*, *lanes 5* and *6*). Cells transfected with pTGF-β1$^{S223/225}$ yielded only monomeric precursor forms (*lane 7* in Figs. 3*B*, 4*A*, and 4*B*), yet mature TGF-β1 was still proteolytically cleaved from pro-TGF-β1$^{S223/225}$ and formed the 24-kDa dimer.

All Mutant TGF-β1 Precursors Generate Biologically Active TGF-β1—Biological activity corresponded well with the amount of 24-kDa mature TGF-β1 detected by immunoblotting (Figs. 3*B* and 4*A*). Following acid activation, supernatants from cells transfected with plasmids pTGF-β1^{S223}, pTGF-β1^{S225}, and pTGF-β1$^{S223/225}$ yielded near wild-type levels of inhibitory activity (Table I). In contrast, pTGF-β1^{S33} transfectants yielded approximately 3-fold more activity than pTGF-β1 transfectants. These observations were consistent among separately performed transfections, with pTGF-β1^{S33} transfected COS cells always generating 3- to 5-fold more inhibitory activity than cells transfected with wild-type plasmid pTGF-β1.

Mutant TGF-β1$^{S223/225}$ Yields Biologically Active TGF-β1 without Prior Acidification—rTGF-β1 synthesized by COS cells is secreted in a latent form (Table I). Similarly, TGF-β1 is released by platelets as a latent high molecular weight complex (26, 27). Activation of rTGF-β1 can be achieved by acidification. Both TGF-β1 and TGF-β1^{S33} were ≥90% biologically inactive prior to acidification, whereas TGF-β1^{S223} and TGF-β1^{S225} were ≥80% inactive. However, TGF-β1$^{S223/225}$ was at least 70% active without acid treatment, and in separate experiments (data not shown) levels of activity before and after acidification were equal. This suggests that the pro region of the TGF-β1 precursor is necessary to confer latency of rTGF-β1. Platelet-derived TGF-β1 was recently shown (26, 27) to be noncovalently associated with a complex involving the dimeric precursor pro region and another protein. The TGF-β1$^{S223/225}$ precursor exists only as a monomer (*lane 7* in Figs. 3*B*, 4*A*, and 4*B*); thus, conformational changes resulting from the substitution of both Cys-223 and Cys-225 may not allow the interactions between mature TGF-β1 and its precursor which result in latency.

DISCUSSION

Previous studies (20) have shown that CHO cells expressing wild-type simian TGF-β1 cDNA produce a 90–110-kDa precursor form. Subsequent studies (29) have revealed that this complex consists of pro-TGF-β1 (residues 30–390), the pro region of the precursor (residues 30–278) and mature TGF-β1 (residues 279–390) (*a*, *b*, and *c*, respectively in Fig. 2*A*) linked by intersubunit disulfide bonds. Specifically, it was shown that Cys-33 forms a disulfide bond with a least 1 half-cystine residue in mature TGF-β1. In this report, we show that substitution of Cys-33 with a serine residue does not affect the proteolytic processing of prepro-TGF-β1, but does result in the generation of more mature 24-kDa dimer (Fig. 3*B*). This correlates with an approximately 3- to 5-fold increase in bioactivity (Table I) and appears to be the result of the dissolution of the 90–110-kDa complex. Apparently, pTGF-β1^{S33} transfectants generated more mature 24-kDa dimer, because the pro region of the TGF-β1^{S33} precursor was unable to disulfide bond to processed mature TGF-β1.

In addition, we demonstrated that the other 2 cysteines located in the pro region at amino acid positions 223 and 225 form interchain disulfide bonds. Substitution of either of these cysteines with a serine still resulted in the formation of the wild-type 90–110-kDa protein; however, some monomeric precursor forms were also present (Fig. 4). Substitution of both Cys-223 and Cys-225 resulted in the production of only monomeric precursor forms, yet mature TGF-β1 was still proteolytically cleaved from the precursor and able to form a bioactive dimer. This maturation appeared to be as efficient as that of the wild-type TGF-β1 precursor.

Although the 3 cysteines located in the pro region of the TGF-β1 precursor appear nonessential for the production of mature rTGF-β1, they may be important in the regulation of TGF-β1. Although dimerization of the precursor seems unnecessary for proteolytic cleavage of mature TGF-β1, it may be required for latency. Cells transfected with a plasmid coding for preproTGF-β1$^{S223/225}$ released TGF-β1 in an active form (Table I). Recently (26, 27), latent TGF-β1 has been purified from human platelets as a high molecular weight complex in which the dimeric pro portion of the TGF-β1 precursor is disulfide-linked to an unknown protein and noncovalently associated with mature TGF-β1. In the CHO cell expression system, there is no evidence for another protein which could form a complex with the precursor and mature forms of TGF-β1 (20, 29). This is also likely to be true in the COS cell system, since the rTGF-β1 precursor and mature forms produced by COS cells appear similar if not identical to those produced by CHO cells when examined by immunoblotting. Our results indicate that mature rTGF-β1 is noncovalently associated with the precursor pro region, and when the pro region cannot form a dimer, latency is not conferred. This is most likely due to the disruption of the noncovalent association. However, at this time, we cannot rule out the possibility that mature TGF-β1 is noncovalently associated with the monomeric TGF-β1$^{S223/225}$ pro region, forming a complex that is not latent because mature sequences necessary for activity are sufficiently exposed.

Recent studies (28) have indicated that carbohydrate structures in the pro region are involved in noncovalently binding TGF-β1 to form a latent complex. Perhaps dimerization of the pro region provides the proper three-dimensional structure and stability for such interactions. Little is known about the regulation of TGF-β1 activity *in vivo*. Since most cell types release TGF-β1 in a latent form (23–25), activation of the latent complex appears to be a critical regulatory step. A clearer understanding of the nature of this latency should aid in determining physiological mechanisms of activation.

The high level of expression and secretion of recombinant TGF-β1 may lead to an unnatural cross-linking, making the disulfide linkage between Cys-33 and half-cystine residues located in the mature polypeptide an artifact of the expression system. On the other hand, it may be indicative of a regulatory role for Cys-33 through interactions with other proteins. This seems especially plausible in view of the disulfide-linked complex isolated from platelets (26, 27).

Thus, the 3 cysteines located in the pro portion of the precursor may influence the maturation and activation of

TGF-β1 in two ways. Cys-223 and Cys-225 allow the precursor to form a dimer and subsequently interact with mature TGF-β1 in a noncovalent manner to form a latent complex, whereas Cys-33 may act by disulfide bonding to mature TGF-β1 and/or other proteins to modulate the action of TGF-β1.

cDNA clones have been obtained for two other TGF-βs, TGF-β2 (37–40) and TGF-β3 (41, 42). The TGF-βs are ~70–80% identical in their COOH-terminal mature regions, whereas the pro regions share only ~30–45% identity. However, the 3 cysteine residues in the pro region are conserved among the three TGF-βs, with TGF-β2 and TGF-β3 each containing additional cysteines in this region. Recombinant TGF-β2 has recently been expressed (43), and as is the case with TGF-β1, acid-activation was required for bioactivity. Experiments are in progress to investigate whether cysteines in the TGF-β2 and TGF-β3 precursors function in a manner similar to those in TGF-β1 and whether their pro regions are also involved in regulating the action of the mature growth factor.

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